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# Chemical stability study of vitamins thiamine, riboflavin, pyridoxine and ascorbic acid in parenteral nutrition for neonatal use

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## Abstract

**Background:** The objective of this work was to study the vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C stability in a pediatric formulation containing high amounts of calcium in the presence of organic phosphate, amino acids, glucose, sodium chloride, magnesium sulfate, pediatric vitamins and trace elements under different conditions using developed and validated analytical methods.

**Methods:** The study was carried out during 72 h with formulations packaged in recommended storage temperature (4°C) and 25°C, with and without photoprotection.

**Results:** The results showed that the methodologies used for assessing the chemical stability of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in the formulation were selective, linear, precise and accurate. The vitamins could be considered stable in the formulation during the three days of study if stored at 4°C. When stored at 25°C vitamin C presented instability after 48 h.

**Conclusion:** The pediatric formulation containing high amount of calcium in the presence of organic phosphate, amino acids, glucose, sodium chloride, magnesium sulphate, pediatric vitamins and trace elements packaged in bag-type trilaminar presented a shelf life of the 72 h, when maintained under refrigeration, between 2°C and 8°C. This shelf life was measured considering the vitamins studied. Further studies are needed including all the vitamins present in this formulation.

## Background

Vitamins are components of parenteral nutrition (PN) used for attending the daily requirements and supplying deficiencies in neonates and, in their majority, are instable [1-3]. The chemical degradation is the most usual cause of vitamin loss in the PN bag. Two reactions are described as the most common and important: oxidation of the ascorbic acid and the reduction of thiamine [4].

The ascorbic acid is the less stable vitamin added to the PN, being rapidly oxidized stimulated principally by high temperatures and catalysed by oligoelements as copper [5-8]. The first step of its degradation route,

when converted to dehydroascorbic acid, is reversible and this compound also has biological activity [9]. Other stages of the route are irreversible and produce compounds without biological activity [10]. The degradation of the ascorbic acid is based directly on the amount of oxygen present in the medium, becoming relevant the packing material used [4-8,11-13]. Several amino acids also interfere in the stability of ascorbic acid not only due its cysteine (cooper ions chelating), but also by reducing its potential, which would decrease the effect of residual oxygen [5-8,14,15]. Another relevant factor related to vitamin C is the oxalic acid formation as degradation final product that has toxic potential and rapidly reacts with free calcium, inducing the precipitation of calcium oxalate [16].

The main route of degradation of the thiamine is caused by its reduction due to the presence of sodium

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metabisulfite, the antioxidant used in the amino acid crystalline solutions [4,7,11,16-18], concentrations of 1 mmol/L, which are sufficient to promote degradation [6]. Riboflavin is one of the vitamins that present more sensibility to photodegradation [7,19]. It is irreversibly converted in lumino-flavin, luminochromo and compounds of less importance in the presence of oxygen [3,4].

A PN formulation comprises large amounts of dissolved components with reactive properties leading to a high potential of incompatibility and instability. The physicochemical instabilities include: formation and precipitation of insoluble salts (i.e. calcium monohydrogen phosphate), degradation or complexation that can alter the bioavailability of the components [20]. These reactions are time-dependant, dependant on the concentration of reactants, pH-value, temperature, light exposure, the presence of catalytically active components and the container material [20].

Although the vitamins stability study in the PN is fundamental due to its importance in the efficacy and clinical security. The vitamins assay methods described in the literature are in majority for determination in biological matrix as blood, serum and maternal milk, or in pharmaceutical forms [1-3,21]. The selective quantification of the vitamins, in this complex matrix (PN) is an analytical challenge [9,22,23]. Ascorbic acid is the vitamin most studied in PN due to its lability. Methods for vitamin C quantification are available in the literature by HPLC [8,11,12], spectrophotometry [6] and titration [24]. They can also be found in the literature method for analysis of vitamin B<sub>1</sub> using HPLC [11,12,19], but the stability of vitamins B<sub>2</sub> and B<sub>6</sub> are less studied and there is no validated method available for quantification of these vitamins in PN. Despite the availability of some methods in the literature there are not official methods for analysis of vitamins in PN, as pharmacopeia. The establishment of an official method is hampered by the heterogeneity of the PN prescribed. Thus, the reliability of a method for analysing a compound in different formulations should be evaluated by the validation. Thus, in this work we developed and validated analytical methods for selectively assay of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in the PN formulation studied.

The objective of this study was to quantify vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in PN formulations in order to investigate the degradation of these vitamins in a pediatric PN containing a high concentration of calcium (93 mg/100 mL) in the presence of organic phosphate (1.1 mEq/100 mL), oligoelements and amino acids, packaged in the trilaminate bag under different conditions.

## Methods

### Formulation preparation

The formulation studied was composed by: 3 g of Pediamino TAU amino acid (Baxter; São Paulo; Brazil), 8,64 g

of glucose 50% (Fresenius; Campinas; Brazil), 4 mEq sodium chloride 20% (Darrow; Rio de Janeiro; Brazil), 4,5 mEq calcium gluconate 10% (Halex Istar; Goiânia; Brazil), 1.1 mmol sodium glycerophosphate 21.6% (Fresenius), 0.25 mEq magnesium sulfate 0.8%, 0.1 mL Ped element (Darrow) and 10 mL MVI 12 opoplex for adult (ICN Farmacêutica; São Paulo; Brazil). The vitamins concentration in PN: 2 mg/mL of vitamin C, 8 µg/mL of folic acid, 1.2 µg/mL of biotin, 0,01 µg/mL of cianocobalamin, 0.03 mg/mL of pantotenic acid, 0.072 mg/mL of B<sub>2</sub>, 0,8 mg/mL of nicotinamin, 0.08 mg/mL of B<sub>6</sub>, 0.06 mg/mL of B<sub>1</sub>, 0.0364 mg/mL of vitamin A, 0.1 µg/mL of vitamin D, 0.2 mg/mL of vitamin E. The final volume of 100 mL was completed with water for injection and packaged in a trilaminate bag with capacity of 300 mL (Halex Istar).

### Reagents and chemical substance of reference

The reagents hydrogen peroxide PA, hydrochloric acid, sodium 1-hexanesulfonate, methanol and glacial acetic acid spectrophotometric grad were purchased from Merck (Darmstadt, Germany). The chemical substance of reference of thiamine hydrochloride (purity: 99.0%) and pyridoxine hydrochloride (purity: 99.9%) were purchased from Roche (Basel, Switzerland). The chemical substance of reference of ascorbic acid (purity: 99.0%) was purchased from Spectrum Chemical (New Brunswick, USA) and the riboflavin 5-fosphate sodium dehydrated (purity: 99.2%) from Merck. Other reagents were obtained from local suppliers.

### Formulation preparation

The PN admixture formulas for neonatal use were prepared aseptically in a 300-mL 3-layered bag composed by polyester, polypropylene and polyethylene (HalexIstar, Goiânia, Brazil) under a laminar-flow hood in accordance with the National Health Department specifications Nr. 272 (1988), designed for infusion through central access. The PN admixture formulas were prepared with market products from pharmaceutical industries and based on official regulations [25-29].

The choice of composition was based in the literature recommendations of preterms [26]. For micronutrients, today, there are controversies regarding the optimal dose of vitamins for preterms. A standard supplementation is often not enough to compensate this patient. It is necessary to use pharmacological doses that exceed the recommendations of the literature [30]. Because of this evidence, the concentrations of the vitamins were overestimated.

Each admixture was prepared separately in triplicate or quadruplicate (referred to as 3 or 4 lots) divided into two at different temperatures: 4°C ± 2°C, in refrigerator; and in 25°C ± 3°C, simulating room temperature. A

different number of samples were tested from each of the lots depending on the technique applied. The experiments were performed on the day of the admixtures preparation and also 24 h, 48 h, and 72 h after preparation, time periods indicated as D0, D1, D2 and D3 respectively.

Samples were aseptically collected from each formulation at appropriate intervals using a plastic syringe for the physicochemical tests.

#### pH evaluation

For the evaluation of pH, a Mettler Toledo potentiometer calibrated with pH 4 and pH 7 buffers was used. For each measurement, a 10 mL sample was collected and placed in an amber glass flask. The pH was measured by dipping the electrode directly into the solution, at room temperature. The pH determination was carried out each day in quadruplicate for each formulation in all the conditions studied.

#### Development of analytical methodologies

The initial analytical conditions for the development of the chromatographic method for vitamins B<sub>1</sub> and B<sub>6</sub> were based in methods described in the literature for other purposes [1,2,21,23]. The wavelength of maximal absorption for each vitamin was determined using individual standard solutions of vitamins B<sub>1</sub> and B<sub>6</sub> (8 µg/mL) for obtaining an absorption spectrum in the UV region. Later, the retention time of each vitamin in the chromatographic system was determined. For the routine analysis, a single standard solution containing the B<sub>1</sub> and B<sub>6</sub> was prepared, using the mobile phase as diluent. The HPLC analysis was realized using diode array detection, by the area under the peak in the wavelength of maximal absorption, 250 and 295 nm for B<sub>1</sub> and B<sub>6</sub>, respectively. For the mobile phase selection, different proportions of methanol: water were tested, 50:50, 40:50, 27:73, 25:75 and 20:80 (v/v). The mobile phase was kept at pH 3.0 using glacial acetic acid. A Shimadzu HPLC system was used equipped with a SPD-M10APDA detector. Data was acquired with Class-VP 6.1 software. The ideal chromatographic conditions obtained for the assay of the vitamins was Bondapak C<sub>18</sub> column size 300 mm × 13 mm, 10 µm (Waters, Milford, USA); mobile phase consisting of a mixture of methanol: water (27:73; v/v) and 1.4% of sodium 1-hexanesulfonate for the ionic pair formation; flux rate of 0.35 mL/min with detection at 250 and 295 nm and 30 µL of injection volume.

The fluorescence is a common phenomenon in aromatic molecules, as vitamin B<sub>2</sub> [31]. Based in the method applied to the raw material vitamin B<sub>2</sub> of the USP a selective and sensitive method to assay B<sub>2</sub> in the PN [28] was developed. Initially, a preliminary study was

realized to set the emission and excitation wavelength to detect the vitamin B<sub>2</sub> in the PN. The emission range was between 400-700 nm, with intervals of 5.0 nm; with excitation at 360 nm, with 2.5 nm of interval; and scan velocity of 1000 nm/min in a Jasco fluorimeter.

Based on the reduction potential of vitamin C, the iodometric titration was applied to assay this vitamin in the PN based in the pharmacopeical methodology for raw material and tablets [23]. The titrant used was iodine 0.05 M SV. The sample was diluted in sulfuric acid 10% (w/v; 25 mL) and a solution of starch 1% (3 mL) and distilled water in sufficient quantity for completing 100 mL were used as indicators. The end point was determined by the formation of a blue coloration. Each mL of iodine 0.05 M VS (volumetric solution) corresponds to 8.806 mg of ascorbic acid [24].

#### Validation of analysis methodologies for assaying vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in the PN

The methodologies used for the assay of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in the PN were validated by the selectivity of the vitamins and their degradation products in the formulation, linearity, precision and accuracy in accordance with recommended procedures [8,32,33].

The specificity of the method for assay of the B<sub>1</sub> and B<sub>6</sub> in the presence of other formulation components of the PN was evaluated by the comparison of the chromatograms obtained from a PN containing the standard vitamins in study (B<sub>1</sub> and B<sub>6</sub>) with PN without the vitamins (placebo). The purity determination of the chromatographic peaks was also used with the software of diode array detector. The specificity of the assay of vitamin B<sub>2</sub> was determined by the comparison between the spectrum of PN with the standard B<sub>2</sub>, placebo and the PN, verifying that the peak observed in the spectrum is attributed to one component alone. For the vitamin C the titration of the placebo was carried out. The results obtained in the placebo were discounted later from the volume obtained with the PN containing the vitamin C.

The linearity was evaluated in three different days, by three vitamins standard with five concentration levels, in the ranges of 20-60 and 30-90 µg/mL (B<sub>1</sub> and B<sub>6</sub>, respectively), 1-5 µg/mL (B<sub>2</sub>) and 50-150 mg (C). The linearity of the method was determined by linear regression analysis of the values obtained experimentally with the *software* Excel<sup>®</sup> (Microsoft, 2002).

The precision of the injection was evaluated in five concentration levels of vitamins B<sub>1</sub> and B<sub>6</sub>: 20-60 µg/mL and 30-90 µg/mL, respectively, in three runs each. The standard deviation (SD) and the relative standard deviation (RSD) were calculated for each point, from the obtained area.

Precision was considered at two levels: repeatability and intermediate precision. It was determined by intra

and inter-day assays. Stock solutions of these vitamins were prepared and aliquots were taken to prepare solutions at three levels of concentration: 80%, 100% and 120% of the sample work concentration. For B<sub>1</sub> (30, 40 and 50 µg/mL); B<sub>6</sub> (45, 60 and 75 µg/mL); B<sub>2</sub> (3, 3.5 and 4.5 µg/mL); and C (80, 100 and 130 mg). The precision of the method was assessed by the SD and RSD of the values obtained experimentally in three days consecutively. The accuracy of the method was verified by determining the known recovery amount of standard vitamins in the spiked PN placebo.

#### Stress stability study of vitamins B<sub>1</sub>, B<sub>6</sub>, and B<sub>2</sub> in the PN

In order to test the selectivity of the developed methodologies for assaying the vitamins studied in PN in the presence of possible degradation products, an accelerated degradation of vitamins, under forced conditions [32] was performed. The assay was carried out separately for each vitamin using the respective analytical method developed. For this, standard solutions were prepared, one with 40 µg/mL of vitamin B<sub>1</sub> and 60 µg/mL of B<sub>6</sub>, and another with 3.5 µg/mL of B<sub>2</sub>. Each vitamin standard solution was stressed using 3% and 10% of hydrogen peroxide for 24 h, at room temperature. After 0, 6 and 24 h of reaction the samples were withdrawn and analysed using the respective methods.

#### Samples preparation

Samples from PN were withdrawn volumetrically to carry out the vitamins assay. For B<sub>1</sub> and B<sub>6</sub> were used aliquots of 7 mL and for B<sub>2</sub> 0.5 mL. Each sample was transferred to a volumetric vessel of 10 mL and the volume completed with mobile phase for B<sub>1</sub> and B<sub>6</sub> and with PN without vitamins for the B<sub>2</sub>.

Vitamin C assay was carried out on a volumetric aliquot of 50 mL. The sample of PN was transferred to an Erlenmeyer containing 25 mL of sulfuric acid 10%, 3 mL of starch solution 1% and distilled water in sufficient quantity for 100 mL. This mixture was immediately titrated with iodine volumetric solution 0.05 M.

#### Statistic Treatment

The experimental results obtained were presented as mean and standard deviation (SD). Comparisons between the results were evaluated by unpaired Student's *t* test with a 95% of confidence limit. Values of *p* < 0.05 were considered statistically significantly.

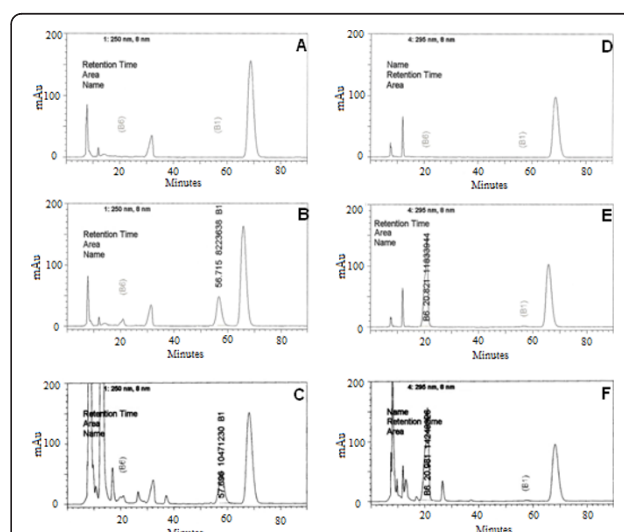
## Results and Discussion

#### Development and validation of analytical methods

The HPLC method was developed by assaying vitamins B<sub>1</sub> and B<sub>6</sub> in the same run. The selectivity of vitamins B<sub>1</sub> and B<sub>6</sub> assay was determined by comparing the chromatograms obtained from placebo, spiked PN with

standard vitamins and samples of PN (Figure 1). The chromatograms show that despite the peaks presence of the other components, the vitamins B<sub>1</sub> and B<sub>6</sub> peaks were satisfactory separated from those others. The peak purity was of 0.9991 and 0.9999 for vitamin B<sub>1</sub> and B<sub>6</sub>, respectively. The peak purity was calculated using the Shimadzu Class-VP 6.1 software.

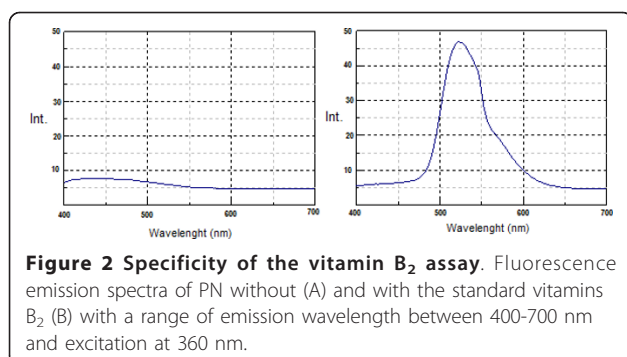
The selectivity of the assay used for the determination of vitamin B<sub>2</sub> in PN was confirmed by the comparison of fluorescence emission spectrums obtained from placebo and PN samples with B<sub>2</sub> in the usual concentration (Figure 2). Three standard curves were also prepared, with five levels each, in the concentration range of 1 to 5 µg/mL, using solution of B<sub>2</sub> in water, spiked placebo with B<sub>2</sub> and PN sample. From these curves could be observed the spectrophotometric profile of the fluorescence light emission from vitamins B<sub>2</sub> and the adequate linearity and selectivity of the method for this vitamin. This was evidenced due to the similarity of the vitamins B<sub>2</sub> in water and spiked placebo with B<sub>2</sub> curves, showing that with the established parameters the others PN components did not interfere in the assay. The curve obtained with the PN sample was above the other curves, but parallel due to the major concentration of vitamin B<sub>2</sub> in this case. For the iodometric titration assay applied for the vitamin C in PN, the selectivity was evidenced by the low volume of the titrant, approximately 0.5 mL, necessary for placebo titration, compared with the volume used for



**Figure 1 Specificity of the vitamin B<sub>1</sub> and B<sub>6</sub> assay.**

Chromatograms of PN placebo (A and D), spiked with the B<sub>1</sub> and B<sub>6</sub> (B and E) and PN containing the multivitamin (C and F) obtained by HPLC at wavelength of 250 and 295 nm, respectively, using column of reverse phase C<sub>18</sub> and a mixture of methanol:water (27:73, v/v) and sodium hexano sulfonate 1,4%, pH 3.0 as mobile phase (*n* = 3).





titration of PN samples containing vitamin C, values between 5.0 and 16 mL.

The linearity relationship was evaluated across a range of each analytical procedure by three standard curves, realized in three different days, as presented in Table 1. The regression lines proved the adequate linearity of the methods (Table 1). The precision was determined by the RSD between the values obtained with the spiked samples with standard vitamins. In accordance with the Table 2, the precision of the methods were demonstrated adequately, since none of the values of RSD found for each level of concentration were less than 5% [28,33]. The system suitability test for HPLC was defined based on the results obtained in several chromatograms. The efficiency column determined by the analyte peak was >2000, the tailing factor <1.5 and the system precision was evaluated by six repeated assays of the same solution, the RSD was less than 2% [28].

The recovery data were obtained from the assay of the known amounts of standard vitamins spiked to the placebo. The obtained values were in a range of 98.6 to 100.8% for all the developed methods, as demonstrated in Table 3. The mean values of the recovery data achieved show that there was no interference of the placebo and the accuracy of the methods was demonstrated.

### Stress degradation study on vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>2</sub> in the PN

In addition to testing the specificity of the methods by the evaluation of PN with and without the standard vitamins, an accelerated study of the vitamins was carried out. This procedure aimed to ensure that the degradation products were not being quantified simultaneously with the studied vitamins. The stress degradation study for vitamin C was not necessary as the method applied is a pharmacopeical methodology.

In the panel A and B of Figure 3 the results of the chemical accelerated stability study of the vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>2</sub> after 6 h of oxidation using hydrogen peroxide 3% and 10% are demonstrated. The content remaining using hydrogen peroxide 3% and 10%, respectively, for B<sub>1</sub> were: 74.7% and 59.8%; for B<sub>6</sub>: 98.4% and 92.4%; for B<sub>2</sub>: 80.6% and 60.9%. The panel C shows the results of the chemical accelerated stability study of the vitamins B<sub>1</sub>, B<sub>6</sub>, B<sub>2</sub> after 24 h of oxidation using hydrogen peroxide 10%. The remaining content of B<sub>1</sub> was of 12.7%; for B<sub>6</sub> 88.9%; and for B<sub>2</sub> 36.7%.

The results obtained by the accelerated degradation tests of the studied vitamins demonstrated the selectivity of the methods developed in the presence of degradation products in PN.

### Determination of the stability of vitamins B<sub>1</sub>, B<sub>6</sub>, B<sub>2</sub> and C in PN

The choice of composition was based in the literature recommendations of preterms [26]. For micronutrients, today, there are controversies regarding the optimal dose of vitamins for preterms. A standard supplementation is often not enough to compensate this patient. It is necessary to use pharmacological doses that exceed the recommendations of the literature [30]. Due to this evidence, the concentration of the vitamins was overestimated.

Figure 4 shows the mean and the standard deviation of the remanent B<sub>1</sub> and B<sub>6</sub> quantity in the formulation until 72 h of storage. The residual quantities after stored at 4°C for B<sub>1</sub> and B<sub>6</sub> were: 96.4% ± 3.1% and 97.5% ±

**Table 1 Regression analysis results of data for assaying vitamins B<sub>1</sub>, B<sub>6</sub>, B<sub>2</sub> and C by HPLC, fluorescence and iodometric titration, respectively**

Features	Vitamins			
	B <sub>1</sub>	B <sub>6</sub>	B <sub>2</sub>	C
Range	20-60 µg/mL	30-90 µg/mL	1-5 µg/mL	50-150 mg
Regression equation <sup>a</sup>	Y = 214695 x + 3168877	Y = 191100 x - 130681	Y = 57.9 x + 28.3	Y = 0.1x + 2.36
SE of intercept	2140.2	694.4	4.5	4.05
SE of slope	48166.4	3445.3	1.9	0.0
Correlation coefficient (r <sup>2</sup> )	0.9984 ± 1.45·10 <sup>-3</sup>	0.9989 ± 1.54·10 <sup>-3</sup>	0.9999 ± 0.00	0.9999 ± 5.0·10 <sup>-5</sup>

<sup>a</sup>y = bx + a, where x is the concentration in µg/mL, except for vitamin C in mg, y is the peak area, for HPLC method, the intensity for fluorescence and volume for titration; a is the intercept and b is the slope. SE= standard error.

**Table 2 Intermediate precision values obtained using the methods for vitamin B<sub>1</sub>, B<sub>6</sub>, B<sub>2</sub> and C analyses in PN**

	Day	80%		100%		120%	
		ID RSD (%)	IT RSD (%)	ID RSD (%)	IT RSD (%)	ID RSD (%)	IT RSD (%)
<b>B<sub>1</sub></b>	1	1.78	1.40	1.89	2.00	0.48	0.88
	2	1.26		0.63		1.06	
<b>B<sub>6</sub></b>	1	0.63	0.90	1.68	1.55	0.41	1.96
	2	1.01		0.95		0.98	
<b>B<sub>2</sub></b>	1	0.40	1.18	1.09	1.59	1.08	1.61
	2	1.17		0.67		0.90	
<b>C</b>	1	0.71	1.46	0.57	1.33	0.43	0.56
	2	0.69		0.55		0.43	

ID RSD means intraday relative standard deviation and IT RSD interday relative standard deviation of 3 measurements average in each day. n = 2 lots.

1.0%; at 25°C with photoprotection: 92.4% ± 3.1% and 93.1% ± 6.0%; without photoprotection: 95% ± 7.6% and 94% ± 5.0%, respectively. The residual B<sub>2</sub> quantity after 72 h of storage at 4°C was: 99.4% ± 1.1%; at 25°C with photoprotection: 94.7% ± 9.2%; and without photoprotection: 99.0% ± 1.6% (Figure 5). Statistically significant variations had not been observed (p > 0.05) for the vitamins during the days of study and under the studied temperatures.

The mean and the standard deviation of the remaining vitamin C percentage within 72 h of storage are presented in the Figure 6. The residual contents after storage at 4°C were: 94.4% ± 2.9%; at 25°C with photoprotection: 85.9% ± 1.1%; and without photoprotection: 87.8% ± 1.3%, showing statistically significant chemical alteration (p < 0.05) of vitamins C in the temperature of 25°C with and without photoprotection during the study.

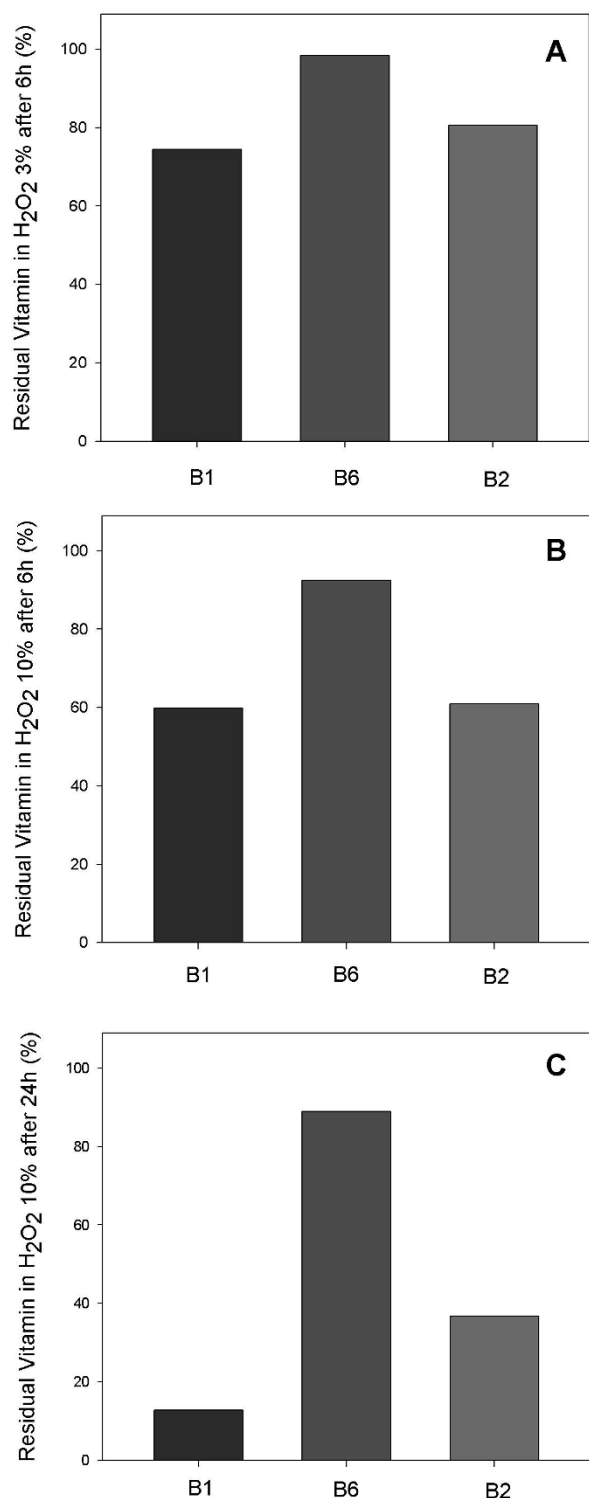
According to ICH, the shelf life of a formulation can be estimated as the period time that the concentrations of the components are not reduced more than 10% [34]. No significant stability alteration (p > 0.05) was observed in the study of vitamins B<sub>1</sub>, B<sub>2</sub> and B<sub>6</sub> during 72 h in all studied conditions, i.e., less than 10% of content loss, as demonstrated in the Figures 4 and 5. However, the vitamin C presented significant alteration (p < 0.05), around 15% of loss after 72 h, when stored at 25°C independent of photoprotection, as demonstrated in Figure 6. These results were expected,

because there was not incidence of direct natural light in the PN studied, only indirect natural light or artificial light. The observed degradation of vitamin C in the formulation could be due to other interfering factors such as the presence of oxygen, the storage temperature and the presence of oligoelements. It is important to reinforce that the important parameter for the variation of the vitamin C loss was the storage temperature, as observed in others studies [6,11] since the PN with and without photoprotection, when kept at 25°C, showed assay values very close and in the storage temperature indicated by the literature, between 2°C and 8°C, no significant alteration in the assay was observed. The loss of vitamin C observed was similar to other studies containing trace elements and vitamins in PN stocked in trilaminar bag [5,7,8]. Moreover, the relative stability of vitamin C is due to the bag used in this study. The trilaminate bag is 100 times less permeable to the oxygen than the EVA material, so the oxidation reaction occurs in minor intensity [23]. However, it has been reported the importance of removing the residual oxygen inside the bag after its preparation [6]. The mean and standard deviation of pH values for PN admixtures during the three days of study, at the two different temperatures selected were measured. In all cases the pH remained around 5.5 throughout the study period and showed no significant differences between the values of D3 and D0 in each condition tested.

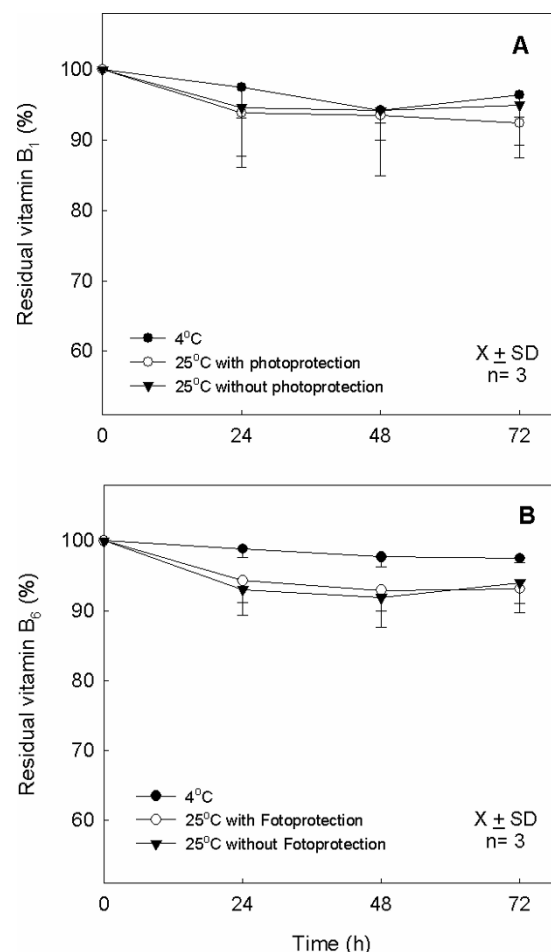
**Table 3 Experimental values obtained in the recovery test of vitamin B<sub>1</sub>, B<sub>6</sub>, B<sub>2</sub> and C by HPLC, fluorescence and titration, respectively**

[B <sub>1</sub> ] (µg/mL)	Mass found (%) ± RSD	[B <sub>6</sub> ] (µg/mL)	Mass found (%) ± RSD	[B <sub>2</sub> ] (µg/mL)	Mass found ± RSD (%)	[C] (mg)	Mass found (%) ± RSD
20	98.6 ± 3.05	30	99.2 ± 1.26	2	98.7 ± 1.35	50	98.7 ± 0.81
30	100.7 ± 2.21	45	100.6 ± 1.97	3	100.7 ± 1.22	80	99.2 ± 1.42
40	98.2 ± 3.82	60	99.8 ± 2.45	3	100.8 ± 1.13	100	98.9 ± 1.19
50	100.2 ± 0.90	75	99.8 ± 1.13	4	100.0 ± 1.27	130	100.0 ± 1.01
60	99.6 ± 0.69	90	99.9 ± 0.58	5	99.6 ± 0.89	150	99.5 ± 0.89

\* Each value represents the mean of 3 analyses.



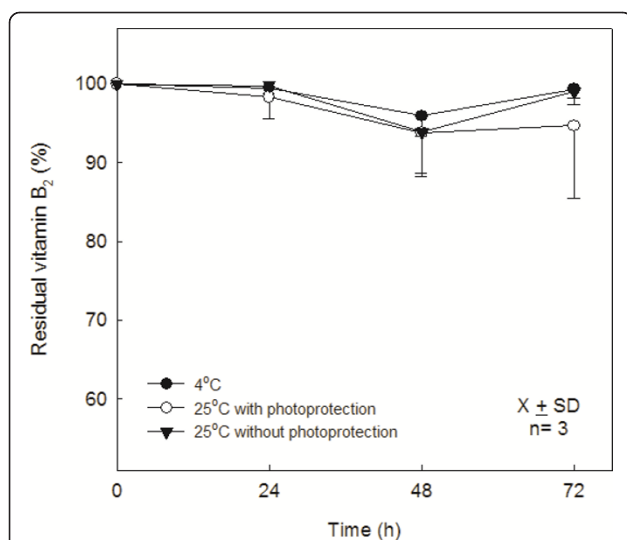
**Figure 3 Stress study of vitamins B<sub>1</sub>, B<sub>2</sub>, and B<sub>6</sub> in PN.** Percentage remaining of vitamins after 6 h of oxidation using hydrogen peroxide 3% (A) and 10% (B) and after 24 h using hydrogen peroxide 10% (C).



**Figure 4 Stability study of vitamin B<sub>1</sub> and B<sub>6</sub>.** Residual content of B<sub>1</sub> (A) and B<sub>6</sub> (B) in PN stored at storage temperatures of 4°C and 25°C with and without photoprotection (n = 3 lots; P > 0.05, D3 compared with D0).

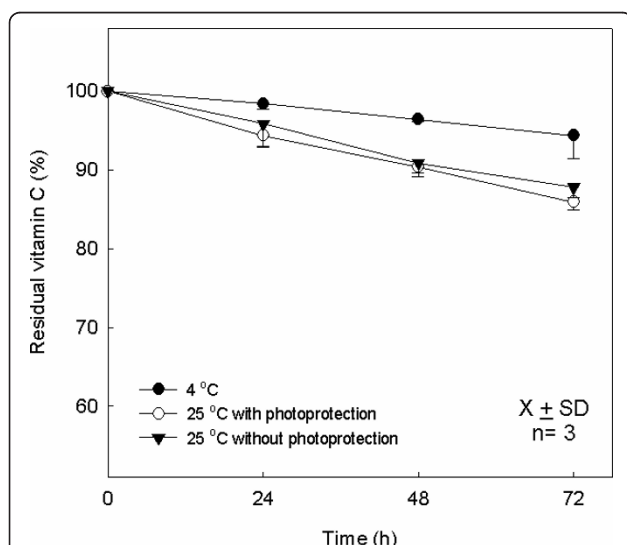
It is established that the pH variation and the presence of reduction agents for vitamin B<sub>1</sub> are factors that contribute to the loss of stability [16]. In the pH range between 5 and 6, the amino acids composition and reduction agents studied did not show significant alteration in the content of this vitamin ( $p > 0.05$ ). It is described in the literature that concentrations of 1 mmol/L of sodium metabisulfite is sufficient to cause degradation of this vitamin and that when this reducing agent is diluted, it does not interfere in this degradation. This fact could be proven, since the PN studied, contained 0.078 mmol/L of sodium metabisulfite, below the quantity described as safe.

The presence of high concentration of calcium associated with organic phosphorus in the presence of oligoelements did not affect the stability of the analyzed



**Figure 5 Stability study of vitamin B<sub>2</sub>.** Residual content of vitamins B<sub>2</sub> in PN stored at 4°C and 25°C with and without photoprotection (n = 3 lots; P > 0.05, D3 compared with D0)

vitamins, with the exception of the stability of vitamin C at 25°C, in which there was a loss of 15% in 72 hours of study. This formulation was chosen due its similarity to *in utero* conditions, which favours greater bone mineralization [35], but with potential risk due to the low final volume and possible interactions leading to loss of vitamins, that, in this formulation was overestimated [4,36-38]. Some works have suggested a possible chemical instability due interaction between vitamins and oligoelements mixed in the same bag. A previous study



**Figure 6 Stability study of vitamin C.** Residual vitamin C in PN at storage temperature 4°C and 25°C with and without photoprotection (n = 3; lots; \*P < 0.05, between the days D0 and D3 at 25°C with and without photoprotection).

demonstrated the compatibility and physicochemical stability of this formulation, but the stability of the vitamins in this formulation remained to be investigated at that time [38]. The lipid emulsion (LE) has a role of photoprotector in the PN, thus its absence favours the degradation of photo sensible vitamins as B<sub>2</sub> and C [7,8,13,16]. So, possibly, the stability of these vitamins will be increased in formulations containing LE.

In spite of PN being an extemporaneous product, knowing the stability characteristics of this formulation makes possible to establish a quarantine period in order to realize effective microbiological control to promote major security in its administration. However, in order to obtain a complete outlook of the chemical stability of the vitamins in this formulation, further studies are needed including all the vitamins present in this formulation, since the PN contains vitamins beyond those studied here.

## Conclusions

The results obtained show that the methodologies used for assessing the chemical stability of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub> and C in the formulation were selective, linear, precise and accurate. The vitamins B<sub>1</sub>, B<sub>2</sub> and B<sub>6</sub> added in a PN containing high concentration of calcium associated with organic phosphorus in the presence of oligoelements could be considered stable during the three days of study, when stored between 4°C and 25°C. The vitamin C presented stability for 48 h at 25°C, with or without photoprotection. Considering the vitamins studied, the shelf life of the formulation studied is 72 h, if maintained under refrigeration, between 2°C and 8°C. Further studies are needed including all the vitamins present in this formulation.

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## Authors' contributions

DOR participated in the design of the study and performed the experiments. DCP carried out the HPLC experiments. LMTRL performed the fluorescence measurements. NMV participated in the design of the study. LMC contributed to the data interpretation and review of the manuscript. VPS conceived of the study and had primary responsibility for writing the manuscript. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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